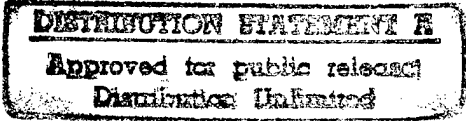


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THESIS

REGULATION OF GnRH RECEPTOR mRNA:
INTERACTION OF GnRH AND ESTRADIOL

Submitted by
Tara Elizabeth Nolan
Cell and Molecular Biology

In partial fulfillment of the requirements
for the Degree of Master of Science

Colorado State University
Fort Collins, Colorado
Spring 1998

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We hereby recommend that the thesis prepared under our supervision by Tara E. Nolan entitled "Regulation of GnRH Receptor mRNA: Interaction of GnRH and Estradiol" be accepted as fulfilling in part requirements for the degree of Master of Science.

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ABSTRACT OF THESIS
REGULATION OF GnRH RECEPTOR mRNA: INTERACTION OF GnRH AND
ESTRADIOL

The integrated functions of the hypothalamus, anterior pituitary gland, and ovaries serve to regulate normal female reproductive function. The hypothalamus secretes Gonadotropin-Releasing Hormone (GnRH), a peptide hormone, in a pulsatile manner. GnRH stimulates the anterior pituitary gland via binding to specific, high-affinity receptors on the plasma membrane of gonadotrophs. GnRH binding initiates a cascade of intracellular events resulting in synthesis and secretion of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and also synthesis and insertion of its own receptors into the plasma membrane. When gonadotropins are released into circulation, they exert their primary biological effects at the gonads. Ovarian hormones, progesterone, inhibin, and estradiol, feedback effects at both the hypothalamus and pituitary gland. Progesterone reduces the pulse frequency and amplitude of GnRH being released from the hypothalamus, thus, decreasing gonadotropin secretion. It is important to note that estradiol does not stimulate secretion of gonadotropins into the circulation. Inhibin is a direct regulator of FSH synthesis and secretion.

A high concentration of GnRH and estradiol, as seen prior to the pre-ovulatory surge, results in an increase in production of gonadotropins and GnRH receptor. The rise in GnRH receptor numbers increases gonadotroph sensitivity

to GnRH and thus the amounts of LH and FSH secreted by the cell in response to GnRH. The mode of GnRH reaching the pituitary gland is, however, critical. In a pulsatile manner GnRH stimulates an increase in GnRH receptors, whereas GnRH delivered via a continuous infusion results in desensitization of gonadotrophs which is characterized by a decrease in the concentration of GnRH receptors. As mentioned above, estradiol increases the numbers of GnRH receptors, presumably through an increase in transcription rates of the GnRH receptor gene. Given that estradiol and GnRH are often combined in therapeutic regimes we asked the question, if estradiol is able to increase GnRH receptor synthesis, will a continuous infusion of GnRH cause GnRH receptor to internalize faster than estradiol can stimulate synthesis?

To address these questions, ovariectomized ewes were administered GnRH continuously via subcutaneous Alzet osmotic mini-pumps for 136 hours, to desensitize the anterior pituitary gland. Twelve hours prior to the end of treatment, half of the ewes were given a bolus intramuscular injection of estradiol. Jugular blood samples were collected for the duration of the experiment and pituitary glands were collected at the termination of the experiment.

The continuous infusion of GnRH caused desensitization of the gonadotrophs. Treatment with estradiol caused concentrations of serum LH to increase in both the saline-treated ewes and in ewes pre-treated with a continuous infusion of GnRH. However, it is important to note the onset of this increase was 4 ½ hours earlier in ewes treated with GnRH. We presume this is

because high levels of circulating GnRH from the pumps was readily available as new GnRH receptors were inserted in to the plasma membrane.

Continuous administration of GnRH caused a decrease in the steady-state levels of mRNA encoding GnRH receptor and number of GnRH receptors. In contrast, treatment with estradiol induced an increase in levels of mRNA for GnRH receptor and number of GnRH receptors relative to controls and in the group receiving continuous GnRH. These results support the conclusion that estradiol, acting on the pituitary gland, stimulates transcription of the GnRH receptor gene. Moreover, desensitization of gonadotrophs by continuous infusion of GnRH and the resulting decrease in mRNA for GnRH receptor and concentrations of GnRH receptors can be overridden by exogenous estradiol. These results support the hypothesis that estradiol may override inhibition of GnRH receptor caused by continuous exposure of gonadotrophs to GnRH, and suggest that new GnRH receptors are synthesized and inserted into the plasma membrane within 6 hours after administration of estradiol.

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TABLE OF CONTENTS

CHAPTER ONE – Review of Literature	1
A. Hypothalamic-Pituitary-Gonadal Axis	1
B. Regulation of pituitary function	3
I. Hypothalamus	3
II. Anterior Pituitary Gland	5
a. Gonadotrophs	7
b. Gonadotropins	8
c. GnRH regulation of pituitary function	10
1. GnRH receptor	10
2. mRNA for LH β , FSH β , and α -subunit	13
3. LH and FSH secretion	14
III. Ovarian Hormones	15
a. Estradiol	16
1. Hypothalamus	16
2. Pituitary Gland	17
3. mRNA for LH β	18
4. mRNA for FSH β	19
5. mRNA for α -subunit	20
6. mRNA for GnRHR and GnRHR concentration	20
7. LH and FSH Secretion	22
b. Progesterone	23
c. Inhibin	25
VI. The Problem: role of GnRH and Estradiol	27
CHAPTER TWO - Regulation of GnRH receptor mRNA	29
A. Abstract	29
B. Introduction	31

C. Materials and Methods	33
I. Animals and Treatments	33
II. Analysis of mRNA	34
III. Hormone Assays	36
IV. Measurement of GnRH receptors	36
V. Statistical Analysis	37
D. Results	37
I. Effect of GnRH on serum levels of LH	37
II. Gonadotropin subunit and pituitary concentration of gonadotropins	42
III. GnRH mRNA and GnRH receptor concentrations	42
A. Discussion	46
B. Implications	49
CHAPTER THREE - Conclusions	51
CHAPTER FOUR - References	53

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CHAPTER ONE

REVIEW OF LITERATURE

A. Hypothalamic-Pituitary-Gonadal Axis

The integrated functions of the hypothalamus, anterior pituitary gland, and ovaries serve to regulate normal female reproductive function. Figure 1 illustrates this integration as a feedback loop (Pierce and Parsons, 1981) termed the "hypothalamic-pituitary-gonadal axis." In particular, the neurosecretory cells located in the preoptic area of the hypothalamus produce Gonadotropin-Releasing Hormone (GnRH) (Wheaton et al., 1978; Braden and Conn, 1991), a peptide hormone that is secreted into the hypophyseal portal circulation (Green and Harris, 1947) for transport to the anterior pituitary gland (Amoss et al., 1971). GnRH binds to receptors on the plasma membrane (Marion and Conn, 1983) of gonadotrophs, thus stimulating the synthesis and secretion of the gonadotropins (Arimura and Schally, 1970; Schally et al., 1972; Clayton and Catt, 1981; Pierce and Parsons, 1981), follicle-stimulating hormone (FSH) and luteinizing hormone (LH), as well as synthesis of GnRH receptor (GnRH receptor).

In the female, as reviewed by Gharib et al. (1990), when gonadotropins are released into the circulation, they exert their primary biological effects at the

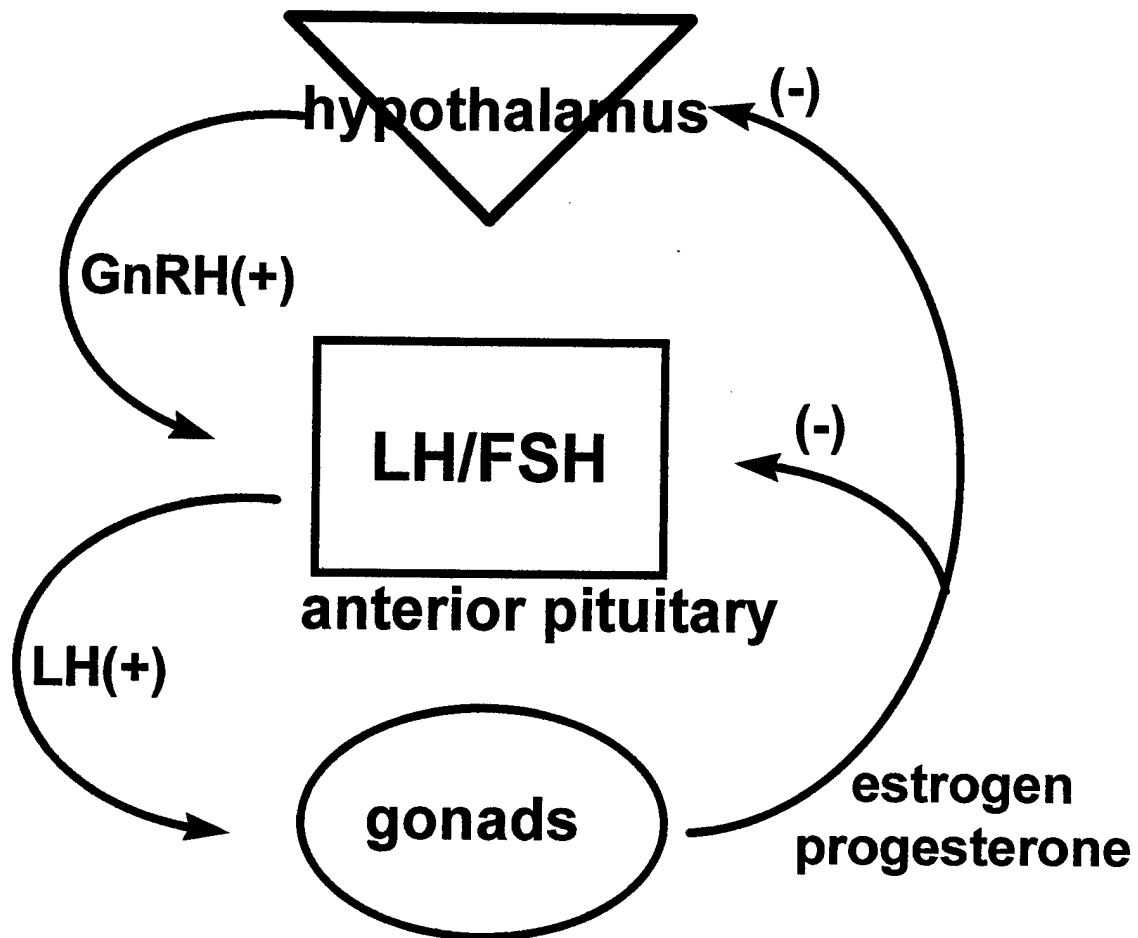


Figure 1. Hypothalamic-pituitary-gonadal axis. GnRH from the hypothalamus stimulates LH and FSH secretion from the anterior pituitary gland. LH and FSH stimulate gametogenesis and steroidogenesis at the level of the gonads. The gonadal steroid hormones, estrogen and progesterone, then typically act in a negative feedback fashion to suppress further production of the gonadotropins

ovary, regulating steroidogenesis and gametogenesis (Gharib et al., 1990). Follicle-stimulating hormone regulates early stages of follicular development (Gharib et al., 1990) whereas, LH regulates later stages of follicular development as well as induces the luteal phase of the estrous cycle by stimulating ovulation and formation of the corpus luteum (Gharib et al., 1990).

The feedback effects of gonadal hormones to regulate hypothalamic and anterior pituitary gland function (Pierce and Parsons, 1981; Karsch et al., 1987; Gharib et al., 1990; Kaiser et al., 1997) is evident in the regulation of GnRH receptor and the gonadotropins themselves. Three hormones are of interest to this regulation; progesterone, inhibin, and estradiol. The latter hormone has the ability to transverse the gonadotroph plasma membrane, bind to an intracellular receptor, and stimulate the synthesis of GnRH receptor. Thus, an increased pulse frequency of GnRH and a high concentration of estradiol result in an increased production of gonadotropins and GnRH receptor.

B. Regulation of Pituitary Function

1. Hypothalamus

The hypothalamus lies at the basal part of the diencephalon below the thalamus, forming the walls and lower part of the third ventricle of the brain (Reichlin, 1967). Within the hypothalamus are clusters of neurons that are symmetrically located around the third ventricle (Reichlin, 1967; Jennes et al., 1988). The endocrine hypothalamus consists of neurons which secrete the neurohormones that regulate anterior pituitary function (Reichlin, 1967). Synaptic

contacts from other neuronal elements connect the endocrine hypothalamus to the rest of the central nervous system (Jennes et al., 1988). Information flow from other brain centers is relayed to hypothalamic neurons which then secrete their neurohormones into the pituitary portal vasculature of the median eminence. Hypothalamic hormones stimulate the endocrine cells in the pituitary to release their specific hormones (Reichlin, 1989; Schally, 1978). Of particular interest in regulation of reproductive function is the hypothalamic hormone, GnRH.

First isolated and characterized as a hypothalamic releasing factor in 1971 (Matsuo et al., 1971), GnRH is a decapeptide hormone (pyroGlu¹-His²-Trp³-Ser⁴-Tyr⁵-Gly⁶-Leu⁷-Arg⁸-Pro⁹-Gly¹⁰amide)(Schally et al., 1971; Burgus et al., 1971) synthesized mainly in the arcuate nucleus (Wheaton, 1978; Braden and Conn, 1991) of the hypothalamus. GnRH is transported from the hypothalamus to the median eminence where it is released in a pulsatile manner (Carmel et al., 1975) into the portal vasculature (Kochman et al., 1975; Swift and Crighton, 1979; Clayton and Catt, 1981) with a frequency of approximately one pulse per hour (Knobil et al., 1980) during the follicular phase. Secretion of GnRH increases during the follicular phase of the ovine estrous cycle (Clarke et al., 1987; Moenter et al., 1991).

The binding of GnRH to receptors on gonadotrophs is the first and vital step in initiating the cascade of intracellular events (Arimura and Schally, 1970; Schulling et al., 1987; Braden and Conn, 1991) necessary for normal reproductive function. Without this initiation step, regulation of the reproductive

cycle is lost. Perhaps the best example of this is a naturally-occurring strain of mice that harbor a mutation in the GnRH gene that prevents correct synthesis of GnRH (Mason et al., 1986). Normal GnRH stimulates the secretion of LH and FSH as illustrated in figure 2, however, these hypogonadal mice produce non-functional GnRH. Therefore, they neither synthesize nor secrete the gonadotropic hormones resulting in complete lack of normal gonadal function (Aschner, 1912; Brodin, 1945; Westman and Jacobson, 1940).

II. Anterior Pituitary Gland

The hypophysis, or anterior pituitary gland, is located directly below the hypothalamus in the sella turcica of the sphenoid bone (Rhodin, 1974; Reichlin, 1989). The pituitary gland is comprised of two separate lobes: the anterior pituitary gland (adenohypophysis) and the posterior pituitary gland (neurohypophysis).

The anterior pituitary gland is made up of at least five endocrine cell types: somatotrophs, lactotrophs, thyrotrophs, corticotrophs and gonadotrophs.. Each synthesizes and secretes specific hormones which regulate biological processes. Somatotrophs and lactotrophs comprise 70% of the anterior pituitary cells (Reichlin, 1989); somatotrophs synthesize and secrete growth hormone and lactotrophs secrete prolactin (Reichlin, 1989). Thyrotrophs, the largest of the cell types, secrete thyroid-stimulating hormone (Reichlin, 1989). Corticotrophs comprise approximately 10% of the cell types and stimulate the adrenal cortex resulting in glucocorticoid secretion (Reichlin, 1989). The

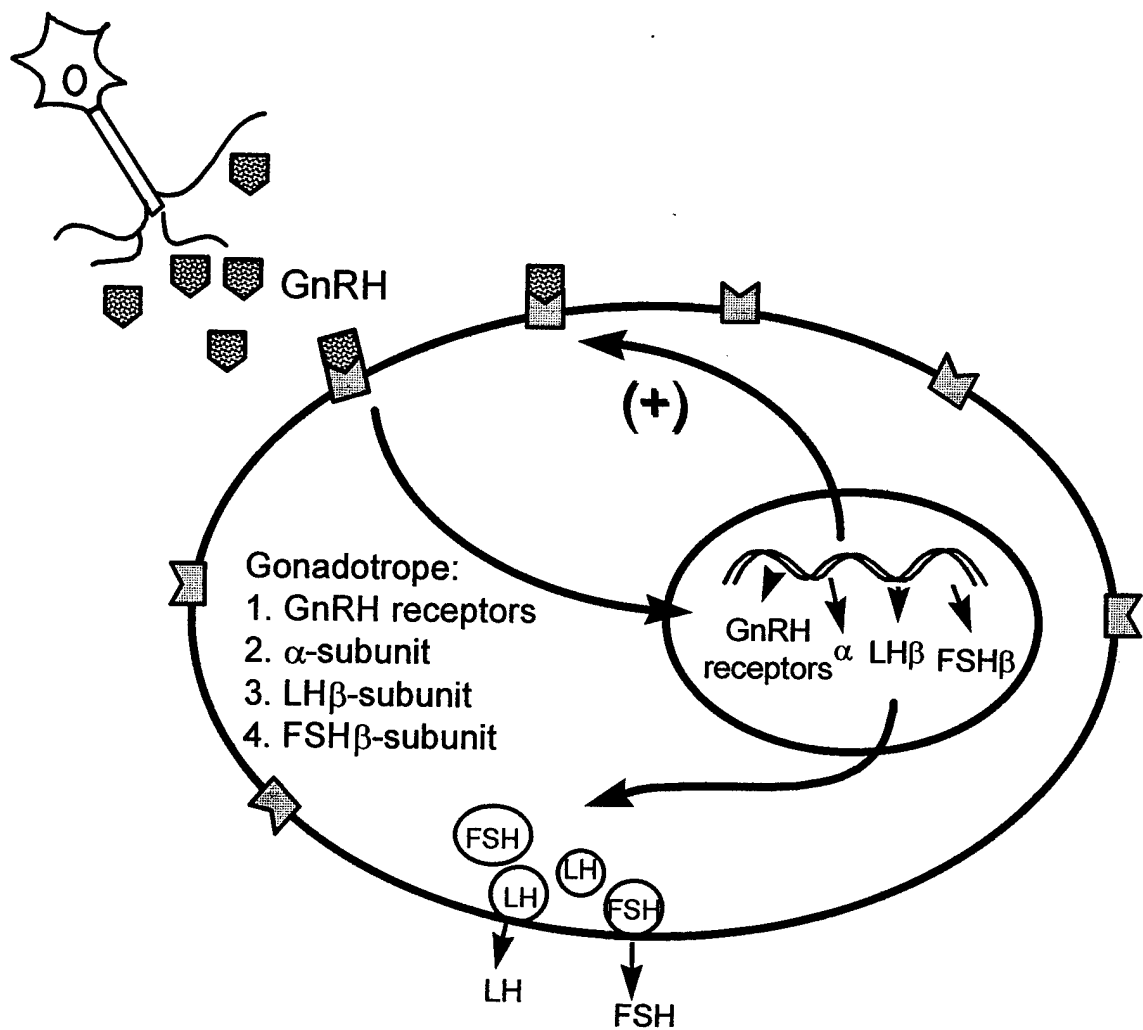


Figure 2. Hypothalamic GnRH binds to specific, high-affinity GnRH receptors located on gonadotrope cells and stimulates expression of the genes encoding the GnRH receptor, the common α -subunit, the LH β -subunit, and the FSH β -subunit. Additionally, GnRH induces the release of the LH and FSH stored in secretory granules.

remaining 8-12% of the cells are gonadotrophs (Ibrahim et al., 1986) which synthesize and secrete the gonadotropins.

a. Gonadotrophs

Gonadotrophs synthesize and secrete LH and FSH (gonadotropins) in response to GnRH. That GnRH exerts its biological effects via a membrane receptor has been long established (Marian and Conn, 1983), however, not until 1992 when cDNAs encoding the GnRH receptor were first reported was there any information as to the structure of this receptor (Reinhart et al., 1992; Tsutsumi et al., 1992). The cDNA for murine GnRH receptor encodes a 327 amino acid protein with 7 membrane-spanning domains (Reinhart et al., 1992; Tsutsumi et al., 1992) which is consistent with membership of this receptor in the superfamily of G-protein coupled receptors (Probst et al., 1992). The GnRH receptor protein structure appears to be conserved across species (Kaiser et al., 1997). Binding of GnRH to the gonadotroph plasma membrane receptor triggers an intracellular cascade resulting in the synthesis of α -subunit, LH β and FSH β -subunits, and GnRH receptor (Marian and Conn, 1983).

The gonadotroph is also regulated by the steroid hormone estradiol which acts via an intracellular receptor (Jensen and Jacobson, 1962; O'Malley et al., 1969; Evans, 1988; Tsai and O'Malley, 1994). Ligand-receptor binding stimulates the gonadotroph to transcribe and translate mRNA for GnRH receptor and to insert GnRH receptors into the plasma membrane. Although some effects of

GnRH and estradiol on gonadotroph functions are similar, these hormones presumably work through functionally different mechanisms. As discussed above GnRH exerts its actions through a G-protein coupled receptor (Marion and Conn, 1983) whereas estradiol acts via an intracellular receptor (Jensen and Jacobson, 1962; O'Malley et al., 1969; Evans, 1988; Tsai and O'Malley, 1994) as illustrated in figure 3.

b. Gonadotropins

As members of the glycoprotein hormone family (Pierce and Parsons, 1981; Jameson and Hollenberg, 1993), LH and FSH are comprised of a specific β -subunit, that is coupled with an α -subunit common to LH, FSH, and TSH (Pierce and Parsons, 1981). Carbohydrates comprise 15 to 30% of the molecular weight of the gonadotropins (Baenziger, 1990). The α -subunit has two asparagine (N)-linked oligosaccharides. The LH β has one and FSH β has two N-linked oligosaccharides (Pierce and Parsons, 1981; Baenziger, 1990).

The α - and β -subunits are each encoded by a single copy gene (Kim et. al., 1988; Gharib et. al., 1989; Watkins et al., 1987; Jameson et. al., 1988) and was initially isolated from humans (Fiddes and Goodman, 1981). In some neoplasms, the α -subunit is produced in the absence of β -subunits (Vaitukaitis, 1979), indicating that there is less stringent control of α -gene expression than β -gene expression (Jameson et al., 1989). In support of this concept, using the OVX-HPD model, Di Gregorio and Nett (1995) showed steady-state levels of

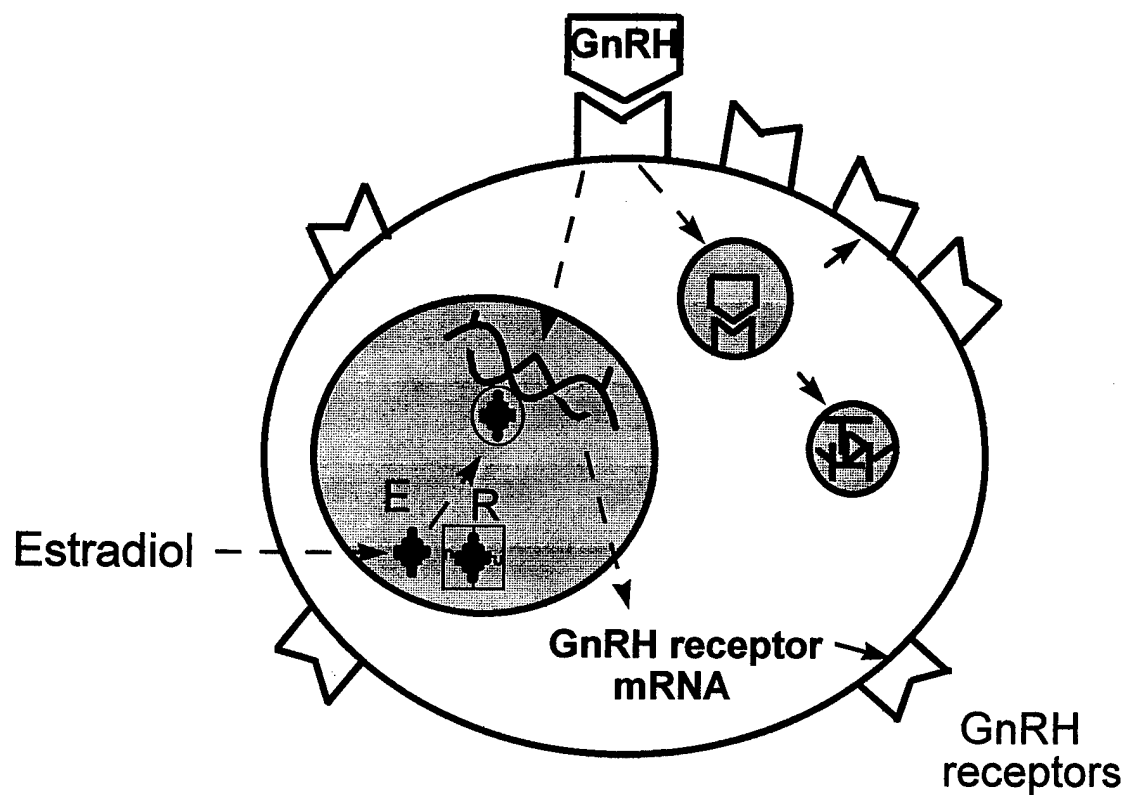


Figure 3. Estradiol is a steroid receptor which binds to an intracellular receptor, inducing a conformational change increasing receptor affinity for the DNA. Estradiol stimulates gene expression of α -subunit, LH β -subunit, FSH β -subunit and GnRH receptor via a mechanism independent of GnRH.

mRNA for α -subunit were not directly regulated by estradiol at the level of the pituitary gland. However, the β -subunits of the gonadotropins appear to be directly negatively regulated by estradiol (Alexander and Miller, 1982; Phillips et al., 1988; Sakurai et al., 1996; Herring et al., 1991; Nett et al., 1991). This indicates that the β -subunit not only lends specificity to the gonadotropins but is a rate-limiting step in LH and FSH protein synthesis.

c. GnRH regulation of pituitary function

GnRH binding to its receptor in the pituitary gland causes release of LH and FSH into the circulation. Thus, the effects of GnRH on gonadotrophs can be studied by monitoring LH secretion (Miller et al., 1990). The sensitivity of the pituitary gland depends on the concentration of GnRH receptors (Miller et al., 1990). The amount of GnRH secreted by the hypothalamus will affect pituitary responsiveness because GnRH is a homologous regulator of its own receptor (Nett et al., 1981).

1. GnRH receptor

Concentrations of plasma membrane GnRH receptor (Clarke et al., 1983; Hamernik et al., 1986; Esbenshade et al., 1986; Culler and Negro-Vilar, 1986; Clarke et al., 1987; Hamernik and Nett, 1988; Gregg and Nett, 1989) and mRNAs for gonadotropin subunits maintained by GnRH (Hamernik and Nett, 1988) are proportional to the frequency and amplitude of the GnRH pulses

(Papavasiliou et al., 1986). In response to GnRH stimulus, GnRH receptors are inserted or removed from the plasma membrane of gonadotrophs (Nett et al., 1984). The sensitivity of the pituitary gland is based on the concentration of GnRH receptor such that more receptors means the pituitary gland can have a greater response to a GnRH challenge. Estradiol stimulates an increase in frequency and amplitude of GnRH pulses during the follicular phase of the estrous cycle. Conversely, during the luteal phase, high levels of progesterone negatively regulate the hypothalamus and pituitary gland, decreasing the frequency of GnRH pulses which, in turn, leads to a decrease in the concentration of GnRH receptor. In the absence of ovarian steroids, variations in GnRH pulse frequency does not increase the concentration of GnRH receptor (Clarke et al., 1987b). This indicates that, in an intact animal, estradiol enhances the increase in GnRH receptor prior to the pre-ovulatory surge.

Regulation of GnRH receptor numbers is complicated and reflects changes in rates of internalization, recycling, degradation and synthesis. Sternberger and Petralli (1975) reported the presence of GnRH receptor on secretory granule membranes. When secretory granules fuse with the plasma membrane to release LH, GnRH receptor numbers increase (Duello and Nett, 1980; Nett et al., 1981). However, this increase is not sufficient to explain the number of membrane receptors seen during the pre-ovulatory surge.

Also it has been shown that GnRH receptor can be incorporated into the plasma membrane independent of LH secretion (Gregg and Nett, 1989). Thus other mechanisms besides fusion of secretory granules with the plasma

membrane must be in place to increase numbers of GnRH receptors. Typically an increase in the number of GnRH receptors is coupled with an increase in serum levels of LH as one effect of GnRH binding is to stimulate secretion LH. In fact, since the isolation of the cDNA encoding GnRH receptor, several labs have demonstrated that increases in GnRH receptor is associated with increased GnRH receptor mRNA. These data indicate regulation of GnRH receptor concentration occurs at the transcriptional level (Sakurai et al., 1992; Sakurai et al., 1997).

Regardless of the precise mechanism, it is clear that GnRH is an important regulator of its own receptor (Nett et al., 1981), however, the mode by which the pituitary gland is exposed to GnRH is critical. Depriving the pituitary gland of GnRH by hypothalamic-pituitary-disconnection results in decreased numbers of GnRH receptors (Clarke et al., 1987a; Gregg and Nett, 1989). Turzillo et al. (1995a) demonstrated that normal GnRH receptor numbers can be restored through exogenous pulsatile GnRH replacement. Therefore, maintenance of steady-state concentrations of GnRH receptor and mRNA requires consistent GnRH stimulation (Sakurai, 1997, 189).

An experimental model in which the anterior pituitary gland is continuously exposed to GnRH was developed to determine the significance of pulsatile release of GnRH from the hypothalamus. Previously, GnRH has been shown to be a homologous regulator of its own receptor in cycling ewes (Tsutsumi, 1993, 1625; Nett et al., 1981), in anestrus ewes (Khalid et al., 1988) and OVX-HPD ewes (Hamernik and Nett, 1988). It has also been shown that an increase in

GnRH receptor concentration in response to GnRH stimulus may contribute to the pre-ovulatory LH surge (Crowder et al., 1984). However, if OVX ewes are administered a continuous infusion of GnRH, the anterior pituitary gland becomes refractory to GnRH (Nett et al., 1981; Crowder et al., 1986). Although the exact reduction in numbers of GnRH receptors to cause desensitization of the gonadotrophs is unknown, Wise et al. (1984) demonstrated that desensitization does occur with a 50% reduction in GnRH receptor concentration. Thus, GnRH receptor concentration may influence tonic LH release, but maximum LH release is not affected unless receptor concentration drops below 50% (Wise et al., 1984). Desensitization is quickly reversible once continuous GnRH input is discontinued (Crowder et al., 1986). To summarize effects: pulsatile GnRH maintains or restores normal GnRH receptor levels. An absence of GnRH decreases concentrations of GnRH receptor. Likewise, continuous GnRH decreases numbers of GnRH receptor.

2. mRNA for LH β , FSH β and α -subunit

GnRH stimulates the synthesis of mRNA for LH β , FSH β , α -subunit and GnRH receptor. Removal of GnRH stimulus through passive immunoneutralization (Sakurai et al., 1997) of sheep or hypothalamic-pituitary disconnection (Hamernik et al., 1986; Nett, 1990) leads to a decrease in mRNA for LH β , FSH β , and α -subunits and GnRH receptor. As expected exogenous pulsatile GnRH increases concentrations of mRNA encoding ovine gonadotropin

subunits and GnRH receptor (Hamernik and Nett, 1988; Turzillo, 1995) highlighting the correlation between GnRH-stimulated synthesis and secretion of gonadotropins and synthesis of and insertion of GnRH receptor into the plasma membrane (Brooks, 1994). The activity of both the human and bovine α -subunit promoters are stimulated by GnRH (Hamernik et al., 1992) and α -subunit mRNA is induced by GnRH in a dose and time-dependent manner (Windle et al., 1990). There is evidence that the gonadotropin subunit genes do not respond equally to pulsatile GnRH stimulation (Marshall et al., 1991; Kile and Nett, 1994). LH β gene expression increases with rapid GnRH pulses (Papavasiliou et al., 1986) while FSH β gene expression increases in response to slow pulses (Dalkin et al., 1989). The ubiquitous α -subunit does not have a characterized response (Dalkin et al., 1989; Weiss et al., 1990).

3. LH and FSH secretion

Two important determinants of the amount of LH and FSH secreted, as reviewed by Braden and Conn (1991), into the peripheral circulation are the amount of GnRH that reaches the pituitary gland (Caraty et al., 1982; Clarke et al., 1987a) and the concentration of GnRH receptor on the plasma membrane of the pituitary gland (Wise et al., 1984; Crowder et al., 1986; Brooks et al., 1993). Gonadotropins are released into circulation in different amounts at different times during the estrous cycle (Clayton and Catt, 1981). Accordingly, the sensitivity,

i.e. concentration of GnRH receptor in the pituitary gland, is an important determinant of the magnitude of the secretory response (Laws et al., 1990).

GnRH stimulates LH secretion (Hamernik and Nett, 1988, 459) in a dose responsive manner (Adams et al., 1979, 735) and removal of GnRH as in hypothalamic pituitary disconnection leads to a dramatic decrease in serum LH and FSH concentrations (Hamernik et al., 1986; Hamernik and Nett, 1988; Gregg and Nett, 1989). Thus, GnRH is essential for gonadotropin secretion. Similarly, the concentration of GnRH receptor in the pituitary gland has been shown to affect the magnitude of gonadotropin secretory response (Laws et al., 1990).

Unlike LH, the pituitary gland does not maintain a large pool of readily releasable FSH and thus the majority of FSH that is secreted (Miller et al., 1977) is newly synthesized (Miller and Wu, 1981). Synthesis and secretion of FSH in ovine pituitary culture is directly proportional to GnRH binding (Brooks, 1991, 109; Miller et al., 1990). Although FSH secretion is induced by GnRH in culture (Huang and Miller, 1980), it appears that FSH is not as tightly linked to GnRH stimulation *in vivo* (Crowder et. al., 1986; Hamernik and Nett, 1988) because pulsatile delivery of GnRH agonist to ewes did not influence serum concentrations of FSH (Herman and Adams, 1990).

III. Ovarian hormones

The ovary produces several hormones including estradiol, progesterone, and inhibin that interact at the level of the hypothalamus and pituitary gland to regulate gonadotropin synthesis and secretion. With regression of the corpus

luteum, serum concentrations of progesterone decrease, allowing LH pulse frequency to increase (Hauger et al., 1977). This decrease in progesterone concentration stimulates the development of ovarian follicles and leads to increased secretion of estradiol (Hauger et al., 1977) and inhibin (Findlay et al., 1990). Before exposure to high levels of LH, estrogen levels predominate; after the LH surge and during the luteal phase of the cycle, progesterone is the major steroid produced. Gonadal hormones can act directly on the ovine pituitary to cause major changes in GnRH binding and gonadotroph responsiveness to GnRH (Miller et al., 1990). These hormones not only regulate the pulse frequency of GnRH from the hypothalamus but also mRNA levels for LH β , FSH β , and α -subunit and GnRH receptor in the anterior pituitary gland. The latter modulates the sensitivity of the anterior pituitary gland to GnRH. Each of these hormones will be discussed to examine their effect.

a. Estradiol

The follicular phase of the ovine estrous cycle is characterized by rising circulating levels of estradiol (Baird et al., 1978) and increased concentrations of GnRH in the hypophyseal portal blood (Clarke et al., 1987). Estradiol affects hypothalamic secretion of GnRH and can stimulate the anterior pituitary gland independent of GnRH (Gregg and Nett, 1989; Turzillo et al., 1995). The effects of estradiol at the hypothalamus and anterior pituitary gland will be reviewed.

1. Hypothalamus

At the hypothalamus, estradiol has a positive effect, decreasing amplitude but increasing the frequency of pulses of GnRH released from the median eminence (Clarke and Cummins, 1985; Caraty et al., 1989; Clarke, 1988; Moenter et al., 1990). According to Evans et al. (1995), estradiol induces secretion of significant amounts of GnRH between pulses and alters the shape of GnRH pulses by reducing the slope of the rising and falling phases of each pulse. These findings lead to the conclusion that during the pre-surge period in the ewe, estradiol induces a qualitative change in the pattern of GnRH release in addition to stimulating GnRH pulse frequency and reducing pulse amplitude (Evans et al., 1995). Thus, increasing concentrations of estradiol stimulate hypothalamic secretion of GnRH during the pre-ovulatory period (Moenter et al., 1990) which will directly affect the actions of the pituitary gland.

2. Pituitary Gland

The responsiveness of the pituitary gland to GnRH is modulated by the number of GnRH receptors on the plasma membrane of the gonadotrophs and can be monitored by measuring serum concentrations of LH (Nett et al., 1981). Therefore, by stimulating an increase in GnRH receptor concentration, estradiol increases the sensitivity of the pituitary gland prior to the pre-ovulatory surge of LH (Reeves et al., 1971; Gregg et al., 1990; Miller et al., 1990) resulting in an increase in serum levels of LH. Using the hypothalamic pituitary disconnection model to remove GnRH from the system it has been shown that estradiol acts at

the level of the anterior pituitary gland to increase GnRH receptor synthesis and numbers of GnRH receptor at certain phases of the reproductive cycle (Crowder and Nett, 1984; Colin et al., 1996).

Although GnRH is necessary for the secretion of gonadotropins, estradiol alone can directly increase the number of GnRH receptors in the pituitary in sheep (Gregg and Nett, 1989) and hypogonadal mice (Naik et al., 1985a). Nett and Naik found an additive effect of GnRH on estradiol-induced increase in GnRH receptor (Nett et al., 1984; Naik et al., 1985a).

3. mRNA for LH β

Rather than causing a simple increase or decrease of gonadotropins, estradiol has a triphasic effect on mRNA for gonadotropins (Herring et al., 1991). First, during the first few hours of estradiol treatment mRNA encoding α -, LH β - and FSH β -subunits decrease (Herring et al., 1991). During the second phase estradiol has been shown to increase mRNA for gonadotropin subunits (Leung et al., 1988; Landefeld et al., 1985). It has been shown that mRNA for β -subunits of gonadotropins decreases and mRNA for α -subunit remains unchanged during estradiol positive feedback in OVX ewes (Hamernik and Nett, 1988). Finally, the third phase is characterized by a negative effect of estradiol as demonstrated by chronic treatment of OVX ewes with estradiol which decreases the amounts of mRNA for LH β (Nilson et al., 1983; Landefeld and Kepa, 1984; Herring et al., 1991). Nilson et al. suggest that estradiol suppresses the accumulation of

mRNAs for α -subunit and LH β -subunits indicating that glycoprotein hormone assembly may not be limited solely by the rate of accumulation of the β -subunits (Nilson et al., 1983)

Although it would seem logical that a decrease in mRNA subunits would result in a decrease in the pituitary content of gonadotropins, Herring et al. did not find a correlation between pituitary gland content of LH and FSH and subunit mRNAs (Herring et al., 1991). This suggests that the continued high concentration of estradiol may cause a post-transcriptional defect preventing gonadotropin synthesis (Herring et al., 1991). In contrast to Herring et al., (1991), Di Gregorio and Nett (1995) found that estradiol decreased pituitary content of LH and steady-state levels of mRNA for LH β subunit but serum concentrations of LH were not affected (DiGregorio and Nett, 1995). Thus, it appears that estradiol does not influence the GnRH-stimulated mechanism that results in gonadotropin secretion, although estradiol may negatively effect gonadotropin subunit synthesis.

4. mRNA for FSH β

FSH does not appear to be as responsive to estradiol as LH. Estradiol decreases steady-state levels of FSH β mRNA *in vitro* (Counis et al., 1983; Phillips et al., 1988; Alexander and Miller, 1982) but steady-state levels remain unchanged *in vivo* (Di Gregorio and Nett, 1995). Inhibition of transcription appears to be the primary event leading to decreased steady-state levels of

subunit mRNAs and FSH secretion *in vitro* (Miller et al., 1990). After treatment with estradiol, serum concentrations and pituitary content of FSH decreased (Di Gregorio and Nett, 1995).

5. mRNA for α -subunit

Since the gonadotropins are heterodimeric requiring both an α and β subunit to complete the protein, it is important to examine the effects of estradiol on the α subunit. In the absence of GnRH *in vitro*, estradiol inhibits α -subunit gene transcription (Phillips et al., 1988) while *in vivo*, steady-state levels of mRNA for α -subunit in sheep were unchanged by estradiol during a 48 hour treatment (Di Gregorio and Nett, 1995). However, chronic treatment of OVX ewes with estradiol lowers content of α -subunit in the pituitary gland (Nilson et al., 1983; Landefeld et al., 1984; Hall and Miller, 1986). Thus, with long term exposure to estradiol, transcription of α -subunit could be a rate-limiting step in the synthesis of gonadotropins.

6. mRNA for GnRH receptor and GnRH receptor concentration

The concentration of GnRH receptor on the plasma membrane of the gonadotroph is a primary determinant of GnRH responsiveness; therefore, it is important to study how estradiol affects GnRH receptor concentration. Using a hypogonadal model to remove hypothalamic input from the system, it has been shown that estradiol can increase GnRH receptor by direct action on the pituitary

gland in sheep (Gregg and Nett, 1989) and mice (Naik et al., 1985a). Estradiol acts directly at the pituitary gland to increase numbers of GnRH receptor *in vivo* in sheep (Moss et al., 1981; Nett et al., 1981; Crowder and Nett, 1984; Khalid et al., 1987; Clarke et al., 1988; Clarke et al., 1989; Turzillo and Nett, 1995), cattle (Schoenemann et al., 1985), mice (Naik et al., 1985), rats (Clayton et al., 1982; Menon et al., 1985) and monkeys (Adams et al., 1981). Estradiol also act *in vitro* in a dose dependent manner to increase numbers of GnRH receptors (Miller and Huang, 1985; Gregg and Nett, 1989; Gregg et. al., 1990; Laws et al., 1990b).

The increase in GnRH receptor induced by estradiol is dependent on both mRNA transcription and protein synthesis suggesting that estradiol induces expression of the GnRH receptor gene (Gregg et al., 1990). This has been confirmed by Sealfon et al., (1990b), Miller et al. (1993) and Turzillo and Nett (1995).

Experimental treatment of gonadotrophs in culture will cause the number of GnRH receptors in the plasma membrane (Marian and Conn, 1983) to vary. In sheep, receptor numbers range from 500 receptors per cell when treated with mid-cycle concentrations of progesterone (Laws et. al., 1990), to 15,000 to 20,000 receptors, after 48-hour exposure to estradiol-17 β (Gregg et. al., 1990; Laws et. al., 1990; Gregg et. al, 1991). In summary, estradiol induces synthesis of GnRH receptor in ovine anterior pituitary cells (Gregg et al., 1990; Sealfon et al., 1990; Laws et al., 1990; Wu et al., 1994) and this increase appears to be

physiologically important to increase numbers of GnRH receptors during the pre-ovulatory LH surge (Crowder and Nett, 1984).

7. LH and FSH Secretion

Estradiol regulation of gonadotropin secretion is complicated in that it's not purely stimulatory or inhibitory. Copping and Malvin (1976) reported estradiol has a biphasic effect on LH secretion meaning that initially estradiol has a negative effect on LH secretion (Nett et al., 1974) as well as pituitary responsiveness to GnRH (Yen et al., 1974) followed by a positive feedback effect. More recently Herring et al. (1991) described estradiol as triphasic, first decreasing serum concentrations of LH for a few hours, then increasing serum LH levels and depleting pituitary gland stores of LH for the next 10 hours (Crowder and Nett, 1984; Schoenemann et al., 1985; Leung et al., 1988) followed by decrease in serum LH levels that appears to remain constant (Herring et al., 1991) accompanied by a slight recovery of pituitary LH levels (Leung et al., 1988; Landefeld et al., 1985). The response of FSH to estradiol exhibits a triphasic response similar to LH but with a much smaller amplitude (Herring et al., 1991).

In OVX ewes (Karsch et al., 1980; Moss et al., 1981) and castrated rams (Schanbacher and Ford, 1977; Parrot and Davies, 1979), long-term treatment with estradiol decreases secretion of LH and FSH, and suppresses pituitary content of gonadotropins (Moss et al., 1981). Pituitary content of LH is lower during anestrus than during the breeding season in intact ewes (Roche et al.,

1970) and in OVX ewes treated chronically with low levels of estradiol (Nett, 1983). Estradiol decreases FSH secretion (Phillips et al., 1988; Alexander and Miller, 1982).

b. Progesterone

When released from the corpus luteum into circulation progesterone exerts its biological effects at both the hypothalamus and pituitary gland. Progesterone has a negative feedback effect on the hypothalamus causing a decrease in the pulse frequency of GnRH secretion thereby reducing the secretion of gonadotropins LH and FSH into circulation. During the luteal phase and pregnancy higher progesterone levels inhibit GnRH secretion. After luteolysis/parturition progesterone declines allowing an increase in LH secretion (Goodman, 1988).

Progesterone may act independently on the hypothalamus but requires the presence of estradiol to act on the anterior pituitary gland. In intact ewes progesterone suppressed serum concentrations of LH (Moss et al., 1981) suggesting estradiol must be present for progesterone to function. Progesterone's inhibitory effect on LH secretion (Pelletier and Thimoier, 1975) is not mediated via receptors for GnRH or pituitary concentrations of LH, suggesting an influence at higher neural centers (Moss et al., 1981) i.e. the hypothalamus. While the studies above utilize an *in vivo* approach the effects of progesterone to decrease GnRH receptor numbers has also been shown *in vitro* (Laws et al., 1990a). Progesterone decreases the responsiveness of ovine

cultures to GnRH by approximately 70% (Batra and Miller, 1985) and decreases GnRH binding (Laws, 1990, 725).

Progesterone's inhibition of LH release is also influenced by inhibin. Progesterone can completely inhibit the sensitizing action of inhibin on GnRH-stimulated LH secretion but its inhibitory action is dependent on the presence of estradiol *in vivo* (Batra and Miller, 1986; Turzillo, unpub, 2). Progesterone totally counteracted inhibin induction of GnRH binding and GnRH-stimulated LH secretion *in vitro* (Laws et al., 1990a). In the absence of inhibin (which increases GnRH-stimulated LH release), progesterone decreased GnRH binding but did not affect GnRH stimulated LH release (Laws et al., 1990) indicating that progesterone is not affecting LH release directly but rather attenuates the effects of inhibin.

Progesterone increases pituitary concentrations of LH and FSH, decreases mRNA for FSH β and α -subunit but does not affect mRNA for LH β (DiGregorio and Nett, 1995). Estradiol and progesterone can act directly at the pituitary gland of the ewe to regulate steady-state levels of gonadotropin subunit mRNAs independent of GnRH (Di Gregorio and Nett, 1995, 166). However, the effects of progesterone at the pituitary gland require other gonadal inputs such as estradiol (Batra et al., 1986). In contrast progesterone can still apparently act at the hypothalamus (Gasc and Baulieu, 1988; Clarke and Cummins, 1984) in OVX animals. Progesterone has different affects on GnRH receptor *in vivo* and *in vitro*. Chronic administration of progesterone to OVX ewes has no effect on

GnRH receptor (Moss et al., 1981; Hamernik et al., 1987) *in vivo* however this may be because there is no estradiol present. *In vitro*, progesterone decreased the number of GnRH receptor (Laws et al., 1990; Sealfon et al., 1990; Wu et al., 1994) and mRNA for GnRH receptor (Wu et al., 1994). Additionally, progesterone overcomes the positive effect of estradiol and inhibin in combination to decrease GnRH receptor by approximately 87% (Wu et al., 1994). However, it is important to point out that *in vitro* experiments do indicate that progesterone is active in the absence of estradiol.

Progesterone negatively regulates gonadotropin secretion by decreasing the pulse frequency of GnRH released from the hypothalamus (Moss et al., 1981; Karsch et al., 1987). Thus, gonadotroph responsiveness to GnRH, which is mediated by the number of GnRH receptors on the plasma membrane, effects gonadotropin secretion. During the luteal, phase LH secretion is low most likely due to a lower concentration of GnRH receptor on the plasma membrane (Laws et al., 1990). At luteolysis, when serum progesterone levels decrease due to regression of the corpus luteum, the negative feedback effect is removed and amounts of mRNA for GnRH receptor begin to increase (Turzillo et al., 1994). This should result in a rise in serum levels of LH punctuated by the pre-ovulatory surge in the follicular phase. Hamernik et al. (1995) suggested that the effects of progesterone on GnRH receptor expression may result from 1) decreasing serum concentrations of progesterone; 2) increasing serum concentrations of estradiol (Batra et al., 1986a; Batra et al., 1986b) and /or inhibin; 3) increased secretion of GnRH; or 4) a combination of these (Hamernik et al., 1995).

c. Inhibin

Inhibin, a protein hormone, is produced by the granulosa cells in females and, has been shown to be a primary regulator of FSH synthesis and secretion. Inhibin can inhibit FSH release (Gregg et al., 1990) from the pituitary without altering LH release and is partially responsible for the differential release of LH and FSH from the pituitary (Brooks et al., 1992; Kirk et al., 1994). Porcine and bovine follicular inhibins decrease FSH secretion in rat pituitary cultures (Fukuda et al., 1986; Gregg et al., 1990) and ovine pituitary culture (Miller, 1988; Miller et al., 1990; Gregg et al., 1991).

To further support this *in vitro* data (Mercer et al., 1987) demonstrated inhibin decreases FSH β mRNA levels at the pituitary gland in sheep. Although inhibin has a significant effect of FSH secretion and FSH β mRNA; steady-state levels of α -subunit mRNA and LH β remain unchanged (Mercer et al., 1987). Therefore, since steady-state levels of α mRNA are unaffected by inhibin (Miller, 1988), the decrease in FSH secretion should be dependent entirely upon decreased transcription of FSH β mRNA levels unless regulation occurs at translation or post-translationally.

Inhibin, like estradiol, may increase GnRH receptor numbers during the pre-ovulatory period. Miller et al. (1990) demonstrated that inhibin causes a 6.6-fold increase in GnRH binding above control indicating an increase in numbers of GnRH receptor in the pituitary gland. Also, Gregg et al. (1990) showed inhibin

causes an increase in GnRH receptor number. Further, using Scatchard analysis, Gregg et al. (1991) showed that inhibin doubled the number of GnRH receptor. Thus, sensitization of the pituitary gland in vivo results from an increase in receptors.

Treatment with estradiol and inhibin causes the greatest increase in pituitary sensitivity to GnRH and analogs (Miller and Huang, 1985; Laws et al., 1990a; Laws et al., 1990b; Gregg et al., 1990; Gregg et al., 1991). Gregg et al. (1991) concluded that these hormones increase the number of GnRH receptor via separate mechanisms, one by a plasma membrane receptor and the other by an intracellular receptor.

The effects of inhibin to increase GnRH receptor numbers in vitro has led investigators to suggest that both estradiol and inhibin cooperate to increase pituitary gland sensitivity to GnRH during the pre-ovulatory period in (Findlay et al., 1990).

In addition to being secreted into circulation from the follicle, the anterior pituitary gland of rats synthesizes both the α and β subunits of inhibin, although secretion of bioactive inhibin by the anterior pituitary has yet to be demonstrated (Roberts et al., 1992). Thus, there is potential for an autocrine or paracrine effect of inhibin produced by the anterior pituitary gland to induce synthesis of GnRH receptor or effect mRNA for gonadotropin subunits.

VII. The Problem—Role of GnRH and Estradiol

When secreted in a pulsatile mode, GnRH increases concentrations of mRNA for GnRH receptors, presumably via an increase in the rate of transcription, which in turn leads to synthesis of new GnRH receptors which are inserted into the cell membrane. However, if GnRH is administered continuously, as in the case of clinical treatment for endometriosis, uterine fibroid tumors, and breast cancer, GnRH receptors are internalized more rapidly than they are replenished and the pituitary gland becomes refractory to stimulation by GnRH. In most instances it is probably degraded. What is not known is whether this homologous desensitization is mediated only by receptor internalization or whether continuous treatment with GnRH also affects expression of GnRH receptor gene.

Another important modulator of GnRH receptor synthesis is estradiol. Estradiol is a steroid hormone that regulates GnRH receptor synthesis by binding to intracellular receptors, which in turn, interact with DNA. Previous experiments have shown that treatment with exogenous estradiol increases the steady-state levels of mRNA for GnRH receptors and concentration of GnRH receptors.

So, although GnRH and estradiol regulate expression of GnRH receptor, they appear to use different pathways that are probably mediated via different response elements on the DNA. Because these two hormones influence GnRH receptors through independent pathways, it is hypothesized that: One, continuous infusion of GnRH will decrease steady-state levels of mRNA encoding GnRH receptor. And two, since the effects of estradiol are regulated

by a receptor different from that for GnRH, we also hypothesize estradiol will override the negative effects of continuous GnRH infusion on mRNA encoding GnRH receptor.

CHAPTER TWO

REGULATION OF GnRH RECEPTOR mRNA

A. Abstract

GnRH serves as a homologous regulator of its own receptor and can either increase (pulsatile delivery) or decrease (continuous delivery) number of GnRH receptors on gonadotrophs. Estradiol also regulates numbers of GnRH receptor, but always in a positive manner. Because estradiol acts via intracellular receptors, and GnRH exerts its effects through a membrane receptor, these two hormones appear to regulate GnRH receptor by different mechanisms. The objective of this experiment was to test the hypothesis that estradiol will override the negative effect of continuous infusion of GnRH on GnRH receptor gene expression and stimulate de novo GnRH receptor synthesis. OVX ewes were administered GnRH (10 μ g/h, n = 10), to down-regulate GnRH receptor, or saline (n = 10) continuously via subcutaneous Alzet osmotic mini-pumps for 136 h. At 124 h, 5 ewes in each group were administered estradiol (25 μ g IM) and anterior pituitary glands were collected 16 hours later. Blood samples were collected every 4 hours for 120 hours and then every 30 min for the duration of the study. GnRH caused an initial increase in circulating levels of LH with peak levels occurring approximately 5 ½ hours after

beginning infusion, after which concentrations of LH decreased to and remained at pre-treatment levels. Circulating concentrations of LH increased approximately 10 hours after treatment with estradiol in ewes administered saline, whereas, circulating concentrations of LH in ewes treated with a continuous infusion of GnRH increased 4 ½ hours earlier—a more rapid response.

Treatment with GnRH and estradiol reduced levels of mRNA for LH β ($p < 0.01$). Likewise, treatment with GnRH alone reduced mRNA for LH β ($p < 0.005$). Alpha-subunit levels did not change in response to either treatment with GnRH or estradiol. Compared to saline-treated controls, treatment with GnRH alone decreased the amount of FSH β mRNA ($p < 0.05$). Treatment with estradiol also caused a decrease in FSH β mRNA ($p < 0.01$). Levels of FSH β mRNA in ewes treated with both GnRH and estradiol were significantly lower than the control group ($P < 0.0005$).

Neither treatment with GnRH or estradiol affected pituitary concentration of LH or FSH relative to controls. Treatment with estradiol or GnRH plus estradiol decreased pituitary content of FSH relative to treatment with GnRH alone ($p < 0.025$).

Compared to saline-treated controls, treatment with GnRH alone decreased the amount of mRNA for GnRH receptor ($p < 0.01$) and concentration of GnRH receptor ($p < 0.05$) while treatment with estradiol caused a nearly 2-fold increase in GnRH receptor mRNA ($p < 0.05$) and GnRH receptor ($p < 0.01$).

Levels of mRNA for GnRH receptor and numbers of GnRH receptors in ewes treated with both GnRH and estradiol were comparable to those in the control group indicating that estradiol was able to override the negative effect of GnRH by stimulating an increase in mRNA for GnRH receptor and GnRH receptor concentrations. Thus, from these data we conclude that although the gonadotroph becomes refractory to GnRH during homologous desensitization, this desensitization does not affect the cell's ability to respond to estradiol.

B. Introduction

At the level of the anterior pituitary gland, binding of GnRH to GnRH receptor on gonadotrophs initiates the cascade of events necessary for the synthesis and secretion of LH and FSH (Schulling et al., 1976; Braden and Conn, 1991). Sensitivity of the pituitary gland to GnRH is dependent on the concentration of GnRH receptors on the plasma membrane. GnRH and estradiol regulate concentrations of GnRH receptor (Clarke et al., 1988), and therefore, these hormones impact the sensitivity of gonadotrophs and ultimately help regulate gonadotropin secretion. Effects of estradiol on gonadotrophs have been studied in the absence of hypothalamic input; however, it has not been determined if estradiol can override the negative effects of a continuous infusion of GnRH.

In the natural state, cycling ewes receive pulsatile GnRH from the hypothalamus to maintain normal anterior pituitary activity (Karsch et al., 1987; Turzillo et al., 1995a) and continued GnRH stimulation is required to maintain

steady-state concentrations of GnRH receptor (Nett et al., 1984; Hamernik et al., 1986; Sakurai et al., 1996), and mRNA for α -, FSH β - and LH β -subunit (Hamernik and Nett, 1988a; Windle et al., 1990) and mRNA for GnRH receptor (Turzillo et al., 1995; Sakurai et al., 1997). Numbers of receptors is correlated with GnRH-induced LH release (Wise et al., 1984). Therefore, an increase in GnRH receptor in the presence of GnRH leads to an increase in serum concentrations of LH (Gregg and Nett, 1989; Gregg et al., 1991).

GnRH is a homologous regulator of its own receptor (Nett et al., 1981; Khalid et al., 1987; Turzillo et al., 1995a) because depriving the pituitary gland of GnRH by hypothalamic-pituitary-disconnection results in decreased numbers of GnRH receptor (Clarke et al., 1987a; Gregg and Nett, 1989; Nett, 1990; Turzillo et al., 1995) which can be restored to control values by replacement of pulsatile GnRH *in vivo* (Clarke et al., 1987a; Hamernik and Nett, 1988a) and *in vitro* (Kaiser et al., 1993). Pulsatile release of GnRH increases both concentrations of mRNA for GnRH receptor and numbers of receptors (Turzillo et al., 1995a). In contrast, continuous infusion of GnRH leads to desensitization of gonadotrophs (Conti et al., 1977; Nett et al., 1981; Crowder et al., 1986; Mason et al., 1994). This desensitization is characterized by a decrease in the number of receptors (Nett et al., 1981) by approximately 50% (Crowder et al., 1986) and can be identified by measuring serum levels of LH (Nett et al., 1981).

Because endocrine communication between the hypothalamus and pituitary are prevented in the HPD model, it has been shown that estradiol

affects expression of the GnRH receptor gene *in vivo* in the absence of GnRH (Turzillo et al., 1995). Treatment with estradiol consistently increases numbers of GnRH receptors *in vivo* (Moss et al., 1981; Menon et al., 1985; Schoenemann et al., 1985; Gregg and Nett, 1989; Turzillo et al., 1994) and *in vitro* (Laws et al., 1990b; Gregg et al., 1990; Wu et al., 1994).

Based on current research, both GnRH and estradiol contribute to increased concentrations of mRNA for GnRH receptor (Brooks et al., 1993; Turzillo et al., 1994) and maximal expression of GnRH receptor (Crowder and Nett, 1984). So, although estradiol increases the number of GnRH receptors, the mechanism of this increase is unclear. Does estradiol decrease the rate of receptor internalization, increase the rate of recycling, or increase transcriptional rates? If estradiol is able to increase GnRH receptor synthesis, will a continuous infusion of GnRH cause GnRH receptor to internalize faster than estradiol can stimulate new synthesis? The potential for estradiol to override the negative effect of continuous infusion of GnRH on expression of GnRH receptor gene and stimulation of *de novo* GnRH receptor synthesis was outlined with the following objectives: 1) to characterize changes in steady-state concentrations of mRNA for GnRH receptor and numbers of GnRH receptors after down-regulation of anterior pituitary gland with a continuous infusion of GnRH, and 2) to measure effects of estradiol on concentrations of GnRH receptor mRNA and numbers of GnRH receptors after anterior pituitary gland desensitization by GnRH.

C. Materials and Methods

I. Animals and Treatments

Sexually mature, Western range ewes that had been OVX for at least 6 weeks were used in this experiment. Twenty ewes were randomly assigned to four treatment groups. Each treatment group contained five ewes. Ten ewes were administered saline and ten ewes were administered GnRH (10 µg/hr) continuously via subcutaneous Alzet osmotic mini-pumps (Alza Scientific Products, Palo Alto, CA) for 136 hours. At 124 hours 5 ewes receiving saline were given a 25 mg intramuscular injection of estradiol-17β (Sigma, St. Louis, MO) dissolved in safflower oil. Also, at 124 hours, 5 ewes receiving GnRH were given an intramuscular injection of 25 mg of estradiol. At 136 hours, after anesthesia with sodium pentobarbital (29 mg/kg BW, iv.) and exsanguination, anterior pituitary glands were collected from all 20 OVX ewes. Pituitaries were divided midsagittally, immediately frozen on solid CO₂, and stored at -80°C. Jugular blood samples were collected every 4 hours for 120 hours and then every 30 minutes for 16 hours. All procedures involving animals were approved by the Colorado State University Animal Care and Use Committee, and were in compliance with NIH guidelines.

II. Analysis of mRNA

Polyadenylated RNA was prepared from one-half of each pituitary gland (Badley et al., 1988). The integrity of this RNA was verified with Northern blot

analysis of mRNA for GnRH receptor (Turzillo et al., 1994). Steady-state concentrations of mRNA encoding GnRH receptor (Nett et al., 1981), LH β -subunit and α -subunit (Nett et al., 1990), and FSH β -subunit (Hamernik et al., 1987) were quantified. Briefly, 1.5 μ g of poly A+ RNA were immobilized in duplicate on a nylon membrane (Hybond; Amersham, Arlington Heights, IL), cross-linked by exposure to ultraviolet light (Stratagene, La Jolla, CA), and probed with cDNAs for ovine GnRH receptor (Nett et al., 1981), LH β (Nett et al., 1990), FSH β (Hamernik et al., 1987), and α -subunit (Turzillo et al., 1994). The cDNAs were radiolabeled using the random hexamer priming method (Boehringer Mannheim, Indianapolis, IN), and hybridization was performed at 42°C for 24 h in hybridization solution [100% deionized formamide, 10% SDS, denatured, sheared salmon sperm DNA (10mg/ml), 10X Pipes-NaCl-EDTA, nanopure water]. After hybridization, the final wash was in 0.5X SSC (750 mM NaCl and 75 mMNa citrate), 0.1% SDS at 65°C. The nylon membrane was exposed to Hyperfilm MP (Amersham, Arlington Heights, IL) for 4 days (FSH β /GnRH receptor), 7.5 h (LH β), or 8 h (α -subunit). After each autoradiography, the membrane was stripped of the respective cDNA as previously described (Turzillo et al., 1994) and reprobed with radiolabeled poly dT (18mer) which was end-labeled with [32]PgATP (3000 Ci/mmol; Amersham) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Hybridization to radiolabeled dT was performed at 30°C for 1 h in 5x SSC, 10mM NaHPO $_4$, 1mM NaH $_2$ PO $_4$ and 0.12% sarkosyl. Membranes were washed in 2X

SSC at 30°C and exposed to film for 20 min (Juengel et al., 1994).

Autoradiographs were analyzed with the NIH 1.52 Image Analysis Program.

Concentrations of cDNA were normalized to the amount of poly A⁺ RNA in each sample and expressed as arbitrary units.

III. Hormone Assays

Circulating concentrations of LH were determined by radioimmunoassay (Niswender et al., 1969) using NIH-oLH-S24 as reference preparation. Mean limit of detection, intraassay and interassay coefficient of variation (CV) were 127 pg/ml, 11.4% and 11.4%, respectively. Circulating concentrations of FSH (L'Hermite et al., 1972) were determined by radioimmunoassay. Mean limit of detection, intraassay and interassay coefficient of variation (CV) were 6.0 ng/ml, 10.3%, and 10.8%, respectively.

IV. Measurement of Receptors for Gonadotropin-Releasing Hormone

Numbers of GnRH receptors were quantified using a standard curve technique described previously (Nett et al., 1981). Briefly, the concentration of GnRH receptor in a pool of bovine pituitary membranes was determined by Scatchard analyses. A standard curve was generated by incubating increasing quantities of the membrane pool with 0.2 nM [¹²⁵I]D-Ala⁶-GnRH-Pro⁹-ethyl-amide ([¹²⁵I]D-Ala⁶). Partially purified membranes prepared from each experimental pituitary gland were incubated with 0.2 nM [¹²⁵I]D-Ala⁶ in assay buffer [1.21g Tris

Base 10 mM, 147mg CaCl₂ 1 mM, 1g BSA 0.1%] for 4 hours at 4°C. At the end of the incubation, tubes were centrifuged immediately after the addition of 3 mL of ice-cold assay buffer. Amounts of specifically bound [¹²⁵I]D-Ala⁶ in the pellets were compared to the standard curve, and the number of GnRH receptor in each sample was calculated. All samples were quantified in a single assay.

V. Statistical Analysis

Data were analyzed using 1-way Analysis of Variance and when significant treatment effects were observed, means were separated using Least Significant Differences. Pearson correlation coefficient (r) was computed to describe the relationship between concentrations of mRNA for GnRH receptor and numbers of GnRH receptors. Data are presented as mean ± SEM.

D. Results

1. Effect of GnRH on serum levels of LH

GnRH caused serum levels of LH to begin rising immediately, reaching a peak at approximately 5 ½ hours. Then serum levels of LH decreased to remain at levels control ewes for the duration of the experiment (Figure 4). At 124 hours (Figure 5), ½ of the saline treated ewes were given 25 mg of estradiol intramuscularly. This induced an immediate decrease in secretion of LH followed by an increase in secretion of LH beginning approximately 10 hours later. Half of the GnRH treated ewes were also administered estradiol at 124

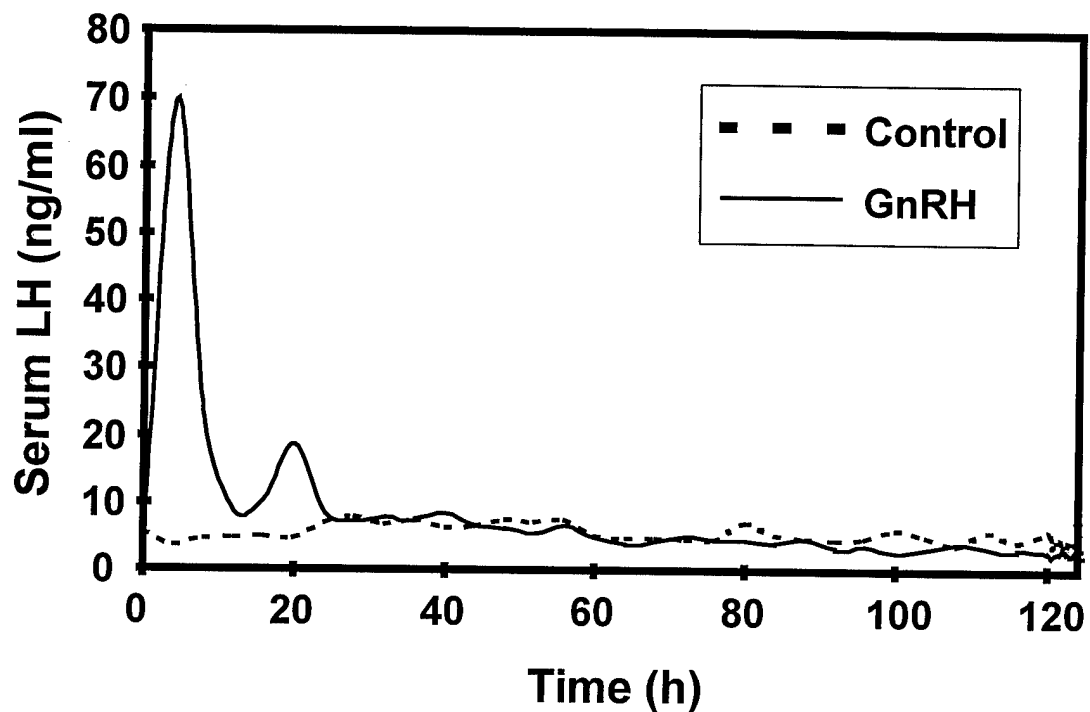


Figure 4. Mean concentrations of LH in serum in OVX ewes. Control ewes (n=10) received saline while 10 ewes received GnRH continuously via subcutaneous Alzet osmotic mini-pumps for 136 h. At 124 h, 12 h prior to pituitary gland collection, estradiol (25 μ g IM) was administered to (n=5 saline treated ewes) and (n=5 GnRH treated ewes). Blood samples were taken every 4 h for 120 h after which sampling frequency increased to every 30 minutes.

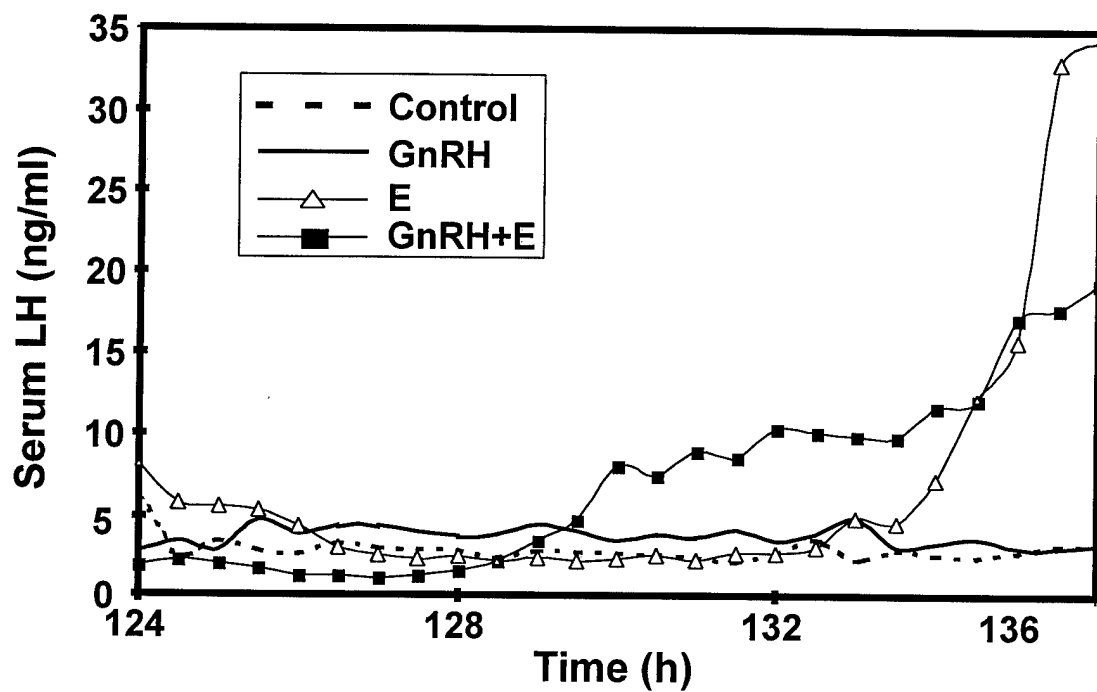


Figure 5. Mean concentrations of LH in serum of OVX ewes from 120 h to 136 h. Control ewes (n=10) received saline, while other ewes were treated continuously with GnRH (n=10). At 124 h, 12 h prior to pituitary gland collection, estradiol (25 mg IM) was administered to saline-treated ewes GnRH-treated ewes. Blood samples were taken every 30 minutes.

hours (Figure 5). There was also a decrease in LH secretion followed by an increase in LH secretion in these ewes in response to estradiol, but it occurred sooner, approximately 4½ hours after the estradiol injection, rather than 10 hours after the estradiol injection as occurred in the saline treated ewes. The magnitude of the release of LH in ewes receiving GnRH plus estradiol was less than in ewes receiving only estradiol. Serum levels of FSH in the treatment groups were not significantly different from control levels (Figure 6).

II. mRNAs for LH β /FSH β / α -subunit and pituitary concentrations of gonadotropins

Treatment with GnRH and estradiol (Figure 7) reduced mRNA for LH β ($p < 0.01$) approximately 59% and in the group treated with GnRH alone mRNA for LH β decreased ($p < 0.005$) approximately 62%. Neither treatment with GnRH or estradiol resulted in a significant variation in α -subunit from control ewes. Compared to saline-treated controls, treatment with GnRH alone decreased the amount of FSH β mRNA ($p < 0.05$) approximately 65%. Likewise, treatment with estradiol also caused a decrease in FSH β mRNA ($p < 0.01$) of approximately 65%. Levels of FSH β mRNA in ewes treated with both GnRH and estradiol were significantly lower than the control group ($p < 0.005$) by 91%.

Estradiol induced a decrease in pituitary concentration of LH ($p < 0.05$) by 72% relative to GnRH treatment (Figure 8). Likewise, estradiol induced a decrease in FSH ($p < 0.02$) by 87% relative to GnRH treatment.

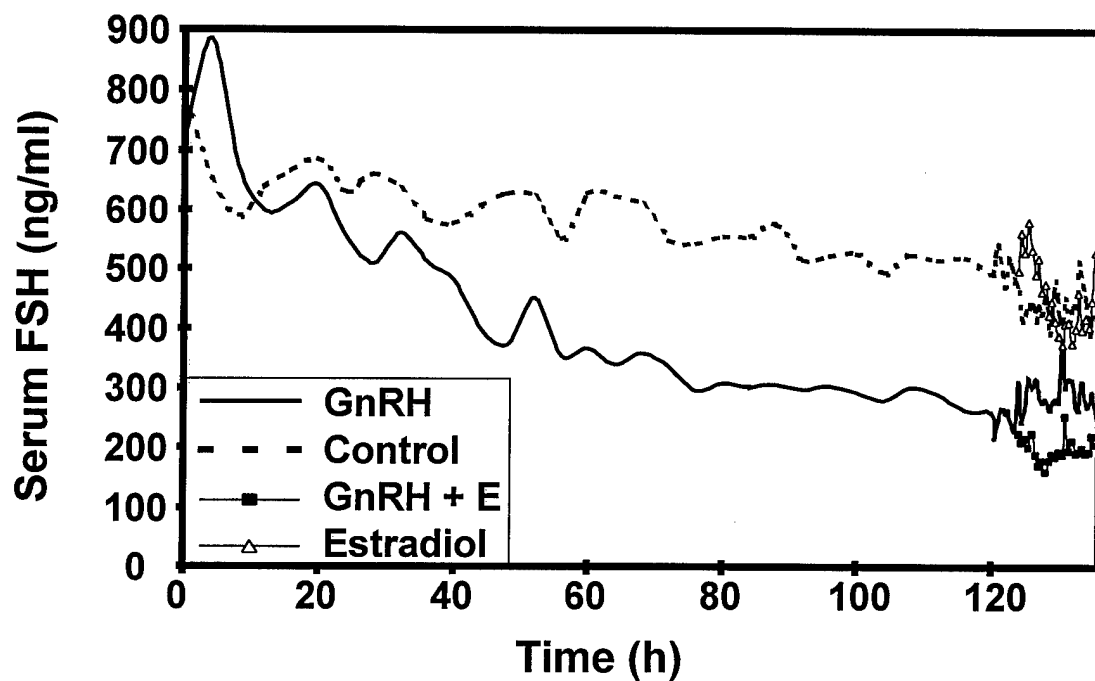


Figure 6. Mean concentrations of FSH in serum of OVX ewes. Control ewes (n=10) received saline via subcutaneous Alzet osmotic mini-pumps for 136 h, while treatment groups received GnRH (n=10) via subcutaneous Alzet osmotic mini-pumps for 136 h. At 124 h, 12 h prior to pituitary gland collection, estradiol injection (25 mg IM) was administered to (n=5 saline treated ewes) and (n=5 GnRH treated ewes). Blood samples were taken every 4 h for 120 h after which sampling frequency increased to every 30 minutes.

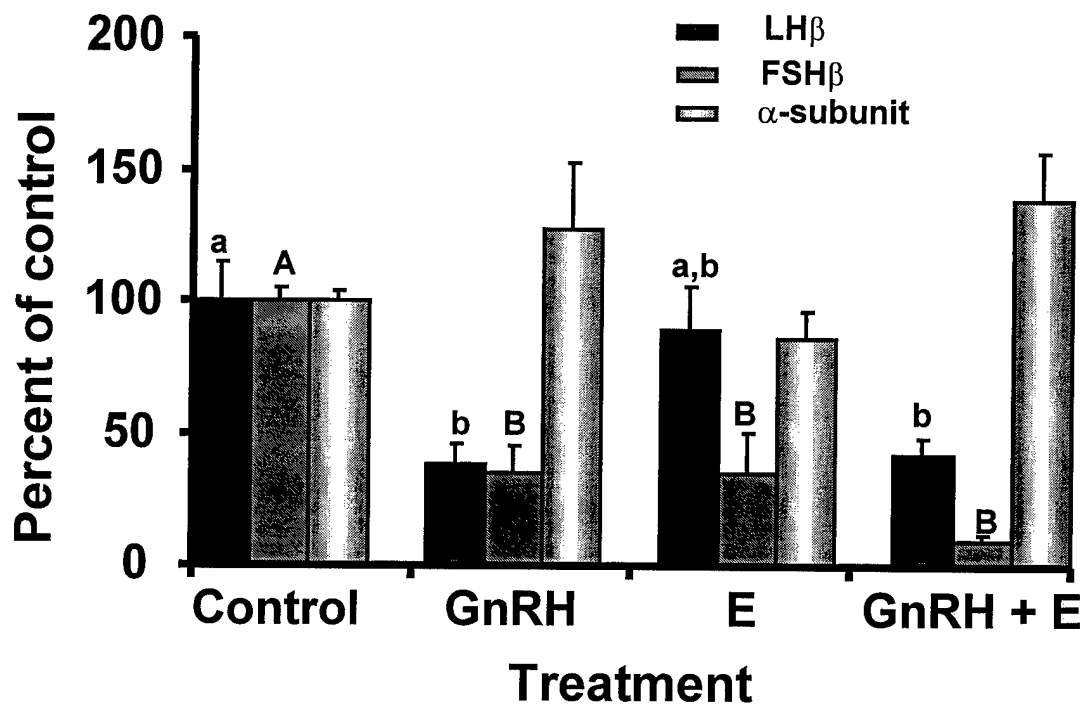


Figure 7. Mean concentrations \pm SEM of mRNA for LH β , FSH β , and α -subunit in pituitary tissue of OVX ewes (n=5/group). Groups were treated with a continuous infusion of GnRH or saline (n=10). $\frac{1}{2}$ of each group was given a 25 mg IM of estradiol 12 h prior to pituitary gland collection. Significant differences among mean concentrations of mRNA for LH β , FSH β , and α -subunit are indicated by letters.

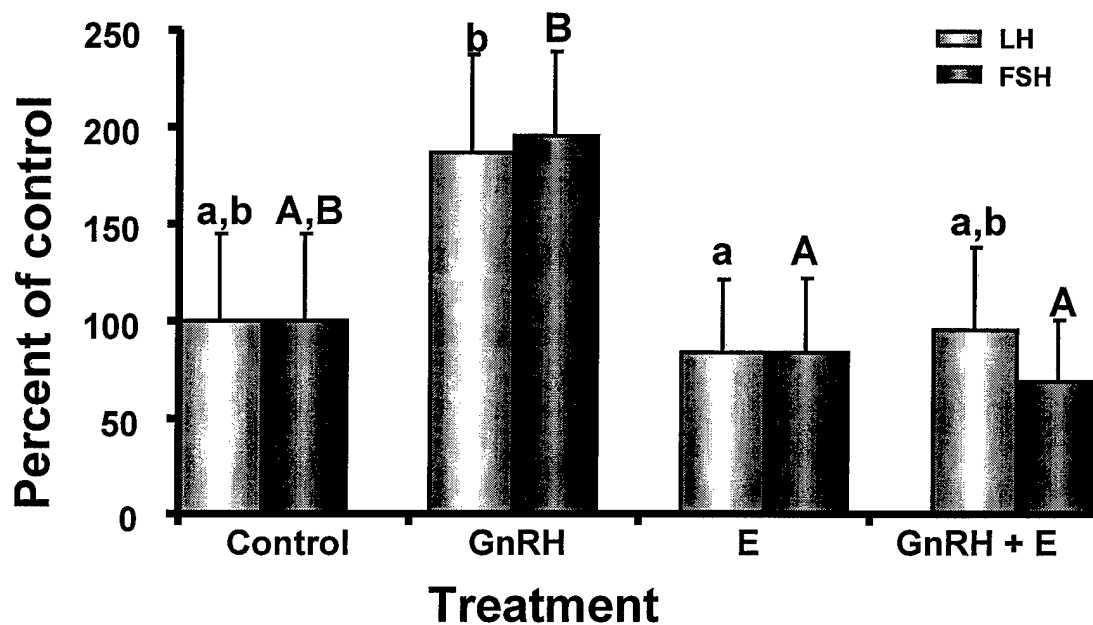


Figure 8 . Mean concentrations \pm SEM of LH and FSH in pituitary tissue of OVX ewes (n=5/group). Groups were treated with a continuous infusion of GnRH or saline (n=10). $\frac{1}{2}$ of each group was given a 25 mg IM of estradiol 12 h prior to pituitary gland collection. Significant differences among mean concentrations of LH or FSH are indicated by letters.

III. GnRH receptor mRNA and GnRH receptor concentrations

Figure 9 illustrates mean concentrations of mRNA encoding GnRH receptor and number of GnRH receptor, represented as percent of control values. Relative to control values, levels of mRNA encoding GnRH receptor in ewes treated continuously with GnRH decreased ($P<0.01$) by 48% and concentrations of GnRH receptor decreased ($P<0.05$) by 69%. Also, in comparison to control values, treatment with estradiol increased the amount of mRNA encoding GnRH receptor ($P<0.05$) by 62% and concentrations of GnRH receptor increased ($P<0.01$) by 89%.

Concentrations of mRNA for GnRH receptor in ewes treated with estradiol and GnRH were elevated in this group relative to levels in ewes treated only with GnRH ($P<0.001$) by 44% . Numbers of GnRH receptors in ewes treated with estradiol and GnRH was also elevated relative to the GnRH treated ewes ($P<0.005$) by 111%. However, concentrations of both mRNA encoding GnRH receptor and concentrations of GnRH receptor in ewes treated with GnRH and estradiol were not different from those in controls. Concentrations of GnRH receptor were correlated to levels of GnRH receptor mRNA (0.78 Pearson correlation, $p<0.0001$).

E. Discussion

Continuous GnRH infusion has been shown to down-regulate the anterior pituitary gland decreasing numbers of hypophyseal receptors for GnRH (Nett et al, 1981; Crowder et al., 1986). Therefore, to validate the first premise of our

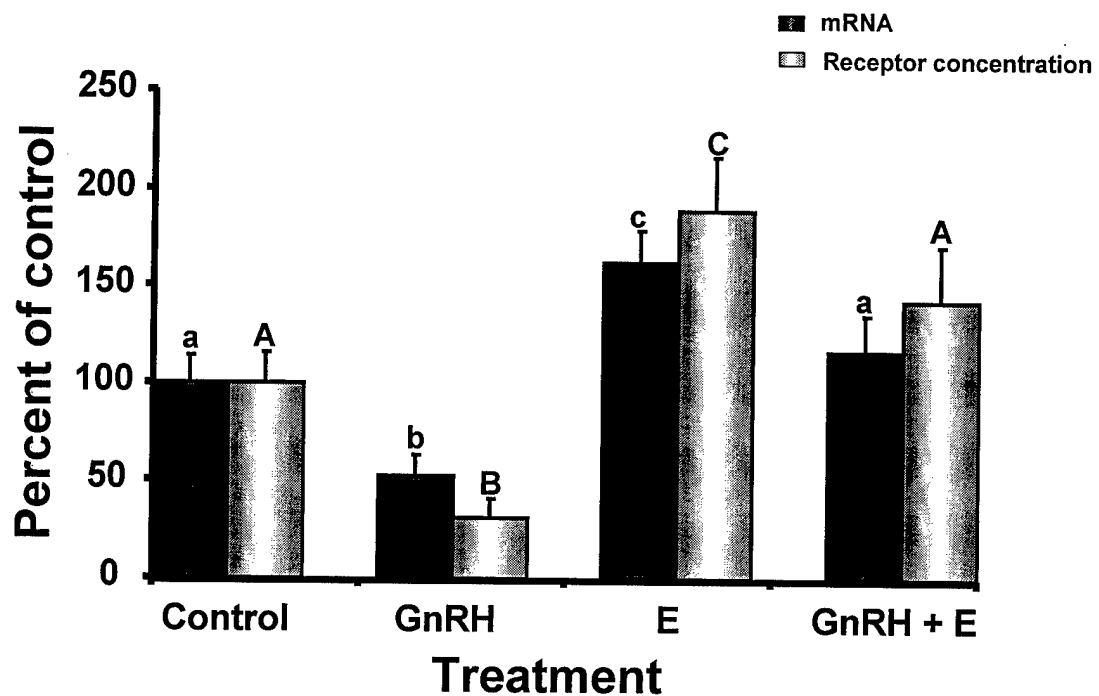


Figure 9. Mean concentrations \pm SEM of GnRH receptor and mRNA for GnRH receptor in pituitary tissue of OVX ewes ($n=5/\text{group}$). Groups were treated with a continuous infusion of GnRH or saline ($n=10$). $\frac{1}{2}$ of each group was given a 25 mg IM of estradiol 12 h prior to pituitary gland collection. Significant differences among mean concentrations of GnRH mRNA or GnRH receptor are indicated by letters.

experimental model, concentrations of serum LH were used to verify that continuous infusion of GnRH caused desensitization of gonadotrophs. As expected, our GnRH treatment induced an LH surge within 5 ½ hours after insertion of implants (Chakraborty et al., 1974; Schuiling et al., 1976; Vizcarra et al., 1997). Following the LH surge, mean concentrations of serum LH in ewes treated with a continuous infusion of GnRH were not different from control serum levels of LH for the duration of the experiment, a previously characterized phenomenon (Nett et al., 1984). Since the GnRH treated ewes continued to be exposed to levels of GnRH that induced a surge of LH, we concluded that gonadotrope desensitization had occurred.

Estradiol has previously been reported to decrease plasma concentrations of LH within 6 hours after treatment (Clarke et al, 1988) followed by an increase in serum concentrations of LH at approximately 16-20 hours (Clarke et al., 1988; Nett et al., 1984). Five of the saline treated ewes that were given estradiol at 124 hours showed a decrease in LH secretion within an hour which was followed by an increase in LH secretion approximately 10 hours later. This matches the effect of estradiol reported by Herring et al. (1991) who found treatment with estradiol decreased serum concentrations of LH from 1-10 hours and then elevated serum levels of LH from 11-22 hours (Herring et al., 1991).

Half of the GnRH treated ewes were administered estradiol at 124 hours. There was also an increase in secretion of LH in these ewes in response to estradiol, but it occurred sooner, approximately 5 ½ hours after the estradiol injection, rather than at 10 hours after the estradiol injection as occurred in the

saline treated ewes. Since estradiol increases the concentrations of GnRH receptors, the ewes receiving a continuous infusion of GnRH were able to respond instantly to an increase in concentrations of GnRH receptors.

Levels of mRNA for FSH β decreased and it appears that a continuous infusion of GnRH has effects similar to removal of GnRH stimulus through immunoneutralization (Sakurai et al., 1997). Treatment with estradiol also caused a decrease in mRNA for FSH β . Levels of mRNA for FSH β in ewes treated with both GnRH and estradiol were significantly lower than the control group indicating an additive negative effect on mRNA synthesis.

Previously, estradiol did not alter mRNA for LH β (Brooks, 1993), however, treatment with GnRH and estradiol, and treatment with GnRH alone both reduced mRNA for LH β . Neither treatment with GnRH or estradiol resulted in a significant variation in α -subunit from control ewes which supports earlier data collected by Di Gregorio and Nett (1995).

Estradiol treatment does not alter pituitary LH concentrations (Clarke et al., 1989) as affirmed by results. It appears that FSH release is directly related to the rate of synthesis (Brooks et al., 1992). Our data indicate that continuous GnRH and estradiol treatment decreased gonadotropin synthesis, however, we found pituitary concentrations of FSH increased in response to a continuous infusion of GnRH.

The first objective of this experiment was to characterize changes in steady-state concentrations of GnRH receptor (Nett et al., 1981; Wise et al.,

1984) and mRNA for GnRH receptor (Turzullo et al., 1995; Sakurai et al., 1997). In accordance with previous data, in our experiment relative to control values, levels of mRNA encoding GnRH receptor and concentrations of GnRH receptor in ewes treated continuously with GnRH decreased, thus confirming desensitization is not solely due to receptor internalization.

The second objective was to measure the effects of estradiol on concentrations of GnRH receptor and amounts of mRNA for GnRH receptor after desensitization by GnRH. Previously, estradiol has been shown to increase GnRH receptor concentrations (Moss et al., 1981; Sealfon et al., 1990; Gregg et al., 1990) and increase steady-state levels of mRNA for GnRH receptor (Wu et al., 1994; Turzillo et al., 1994; Hamernik et al., 1995). Again, in direct comparison with previous publications, treatment with estradiol increased concentrations of GnRH receptor (Clarke et al., 1988; Gregg and Nett, 1989) and the amount of mRNA encoding GnRH receptor (Turzillo et al., 1995) confirming that the stimulatory effects of estradiol on numbers of GnRH receptor in sheep occur directly on the pituitary gland because the continuous infusion of GnRH decreases the sensitivity of the pituitary gland to GnRH from the hypothalamus. However, whether estradiol can override the negative affect of GnRH desensitization on the anterior pituitary gland and increase mRNA for GnRH receptor had not been shown.

Our comparison of estradiol and GnRH treated groups to GnRH only treated groups revealed that estradiol increases expression of GnRH receptor gene in the presence of GnRH because concentrations of GnRH receptor mRNA

were elevated in this group relative to levels in ewes treated only with GnRH.

GnRH receptor was also elevated relative to the GnRH treated ewes. These data show that estradiol is able to increase synthesis of GnRH receptor faster than a continuous infusion of GnRH can cause receptor internalization.

F. Implications

Hypersecretion of luteinizing hormone has been implicated in infertility and miscarriages in women (Risma et al., 1995) and may lead to cyst formation, ovarian tumorigenesis, and infertility (Risma et al., 1995). Since pulsatile GnRH stimulates the gonadotrophs to release gonadotropins, a continuous infusion of GnRH to desensitize the gonadotrophs and prevent LH secretion is used as a method of clinical treatment. As further examples, GnRH can be administered continuously for clinical treatment for endometriosis, uterine fibroid tumors, and breast cancer. This GnRH treatment can elicit menopausal symptoms which may warrant estrogen therapy (Leather et al., 1993). Although the gonadotroph becomes refractory to GnRH during homologous desensitization, this desensitization does not affect the cell's ability to respond to estradiol. Consequently, the down-regulation of mRNA for GnRH receptor and concentrations of GnRH receptor sought through continuous GnRH treatment can be overridden by exogenous estradiol delivered by estrogen therapy. So, where previously, the treatment of continuous GnRH may have been recorded as ineffective; doctors may now understand that the GnRH treatment was most

likely effective but neutralized by estrogen therapy to counteract menopausal symptoms.

CHAPTER THREE

CONCLUSIONS

As discussed previously the continuous infusion of GnRH caused desensitization of the gonadotrophs. Treatment with estradiol caused concentrations of serum LH to increase in both the saline-treated ewes and in ewes pre-treated with a continuous infusion of GnRH. However, it is important to note the onset of this increase was 5 ½ hours earlier in ewes treated with GnRH. We presume this is because high levels of circulating GnRH from the pumps was readily available as new GnRH receptors were inserted in to the plasma membrane.

Continuous administration of GnRH caused a decrease in the steady-state levels of mRNA encoding GnRH receptor and GnRH receptor concentrations. In contrast, treatment with estradiol induced an increase in levels of mRNA for GnRH receptor and GnRH receptor relative to controls and in the group receiving continuous GnRH. These results support the conclusion that estradiol, acting on the pituitary gland, stimulates transcription of the GnRH receptor gene. Desensitization of the gonadotrophs causing a decrease in mRNA for GnRH receptor and concentrations of GnRH receptors can be overridden by exogenous estradiol. These results support the hypothesis that estradiol may override inhibition of GnRH receptor caused by continuous

exposure of gonadotrophs to GnRH, and suggest that new GnRH receptor are synthesized and inserted into the plasma membrane within 6 h after administration of estradiol.

These data are important because GnRH therapy is used to treat several medical conditions. Hypersecretion of luteinizing hormone has been implicated in infertility and miscarriages in women (Risma et al., 1995) and may lead to cyst formation, ovarian tumorigenesis, and infertility (Risma et al., 1995). Since pulsatile GnRH stimulates the gonadotrophs to release gonadotropins, a continuous infusion of GnRH is used to desensitize the gonadotrophs and prevent the pre-ovulatory LH surge. GnRH can be administered continuously for clinical treatment for endometriosis, uterine fibroid tumors, and breast cancer. This GnRH treatment can elicit menopausal symptoms which may warrant estrogen therapy (Leather et al., 1993). Although the gonadotroph becomes refractory to GnRH during homologous desensitization, this desensitization does not affect the cell's ability to respond to estradiol. Consequently, the down-regulation of mRNA for GnRH receptor and concentrations of GnRH receptor sought through continuous GnRH treatment can be overridden by exogenous estradiol delivered by estrogen therapy. So, where previously, the treatment of continuous GnRH may have been recorded as ineffective; doctors may now understand that the GnRH treatment was most likely effective but neutralized by the estrogen therapy used to counteract menopausal symptoms.

Chapter Four

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